(12) UK Patent Application (19) GB (11) 2 294 321 (13) A

(43) Date of A Publication 24.04.1996

(21) Application No 9421118.2

(22) Date of Filing 19.10.1994

(51) INT CL[6]
G01N 33/68 33/577 // G01N 33/543

(52) UK CL (Edition O)
G1B BAE BAG B104 B309 B402 B428 B500 B504 B520 B523
U1S S1313 S2416

(56) Documents Cited
WO 94/15641 A1 WO 91/10683 A1

(58) Field of Search
UK CL (Edition N) G1B BAD BAE BAG BAX
INT CL[6] G01N 33/577 33/68
ONLINE: WPI, BIOTECH/DIALOG

(71) Applicant(s)
Yamanouchi Research Institute

(Incorporated in the United Kingdom)

Littlemore Hospital, OXFORD, OX4 4XN, United Kingdom

Imperial Cancer Research Fund

(Incorporated in the United Kingdom)

Institute of Molecular Medicine, John Radcliffe Hospital, HEADINGTON, Oxford, OX3 9DU, United Kingdom

(72) Inventor(s)
Ian Bird
Julia Spragg
Chris Buckley

(72) cont
David Simmons
Jon Fawcett

(74) Agent and/or Address for Service
Reddie & Grose
16 Theobalds Road, LONDON, WC1X 8PL, United Kingdom

(54) Method of screening for CD31 interaction inhibitors

(57) A method of screening for inhibitors e.g. antibodies of CD31 interactions which comprises incubating labelled CD31 component with potential inhibitor, adding this mixture to CD31 component immobilised on a support, washing and detecting label. Alternatively the method may comprise incubating the potential inhibitor with CD31 component immobilised on a support, adding labelled CD31 component, washing and detecting the label. The homotypic binding of CD31 involves specific engagement of binding sites located in domains 2 and 6.

GB 2 294 321 A

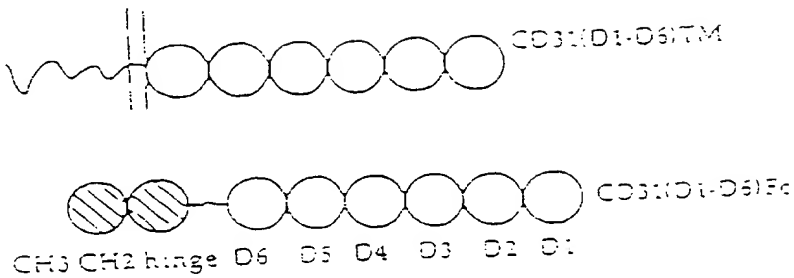

FIGURE 1

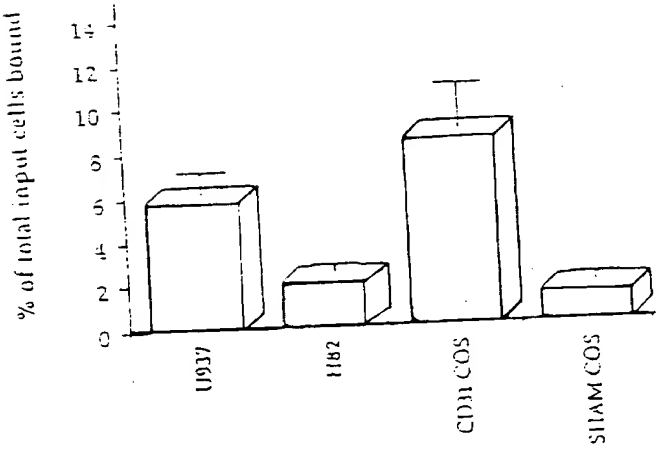

FIGURE 2

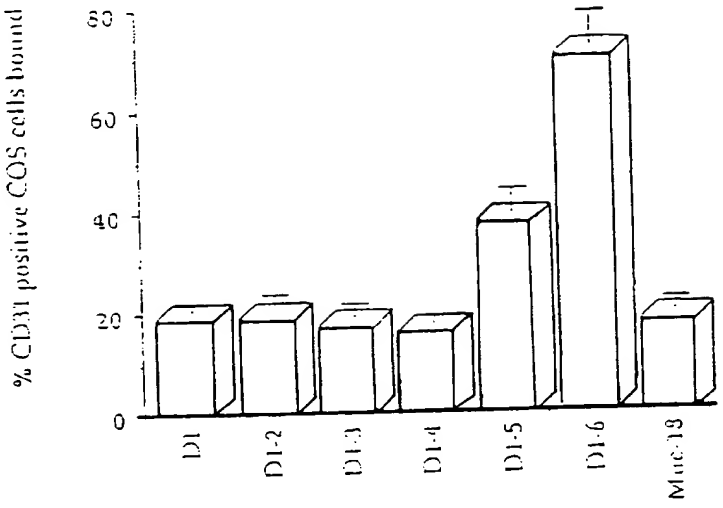

FIGURE 3

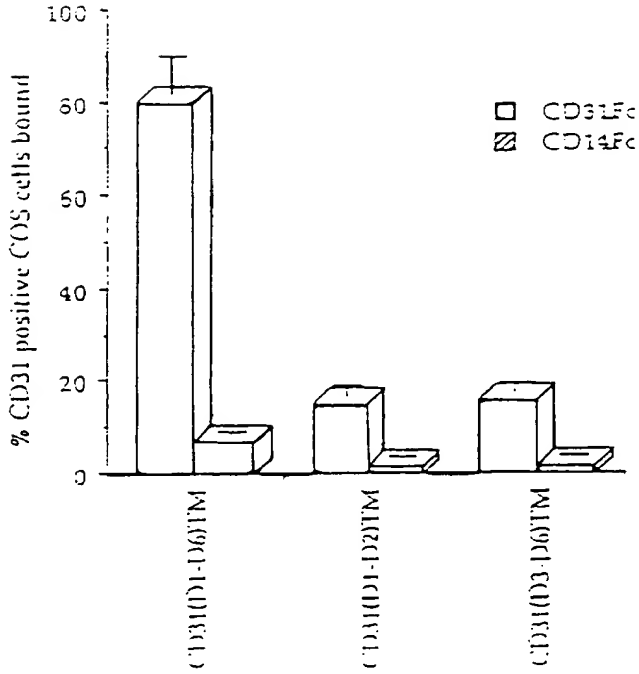

FIGURE 4

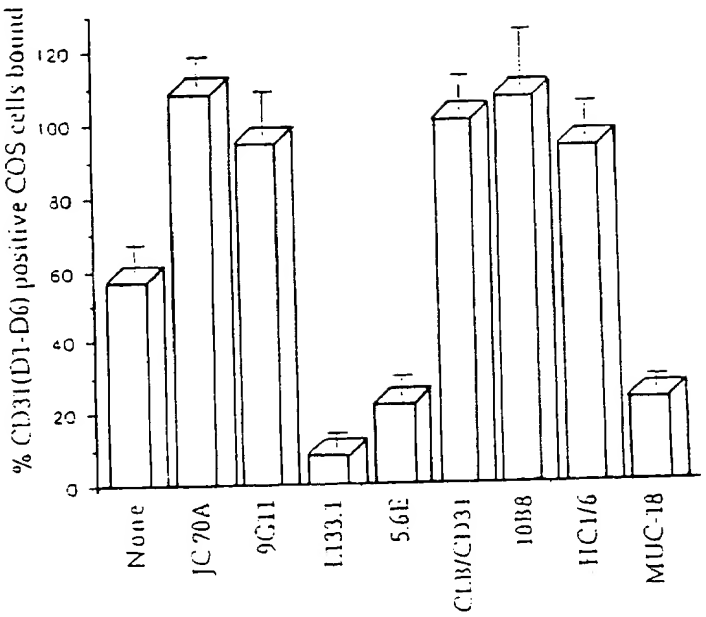

FIGURE 5

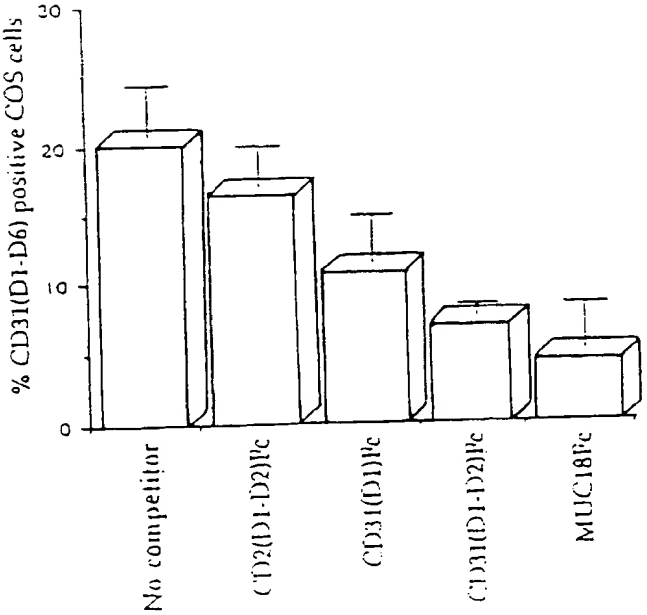

FIGURE 6

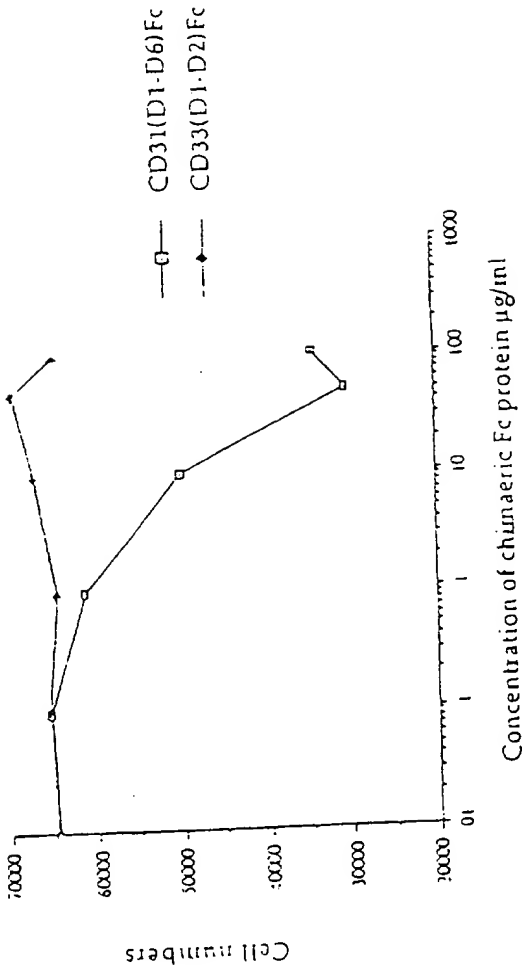
CD31(D1-D6)Fc
CD33(D1-D2)Fc
Cell numbers
Concentration of chimaeric Fc protein µg/ml

FIGURE 7

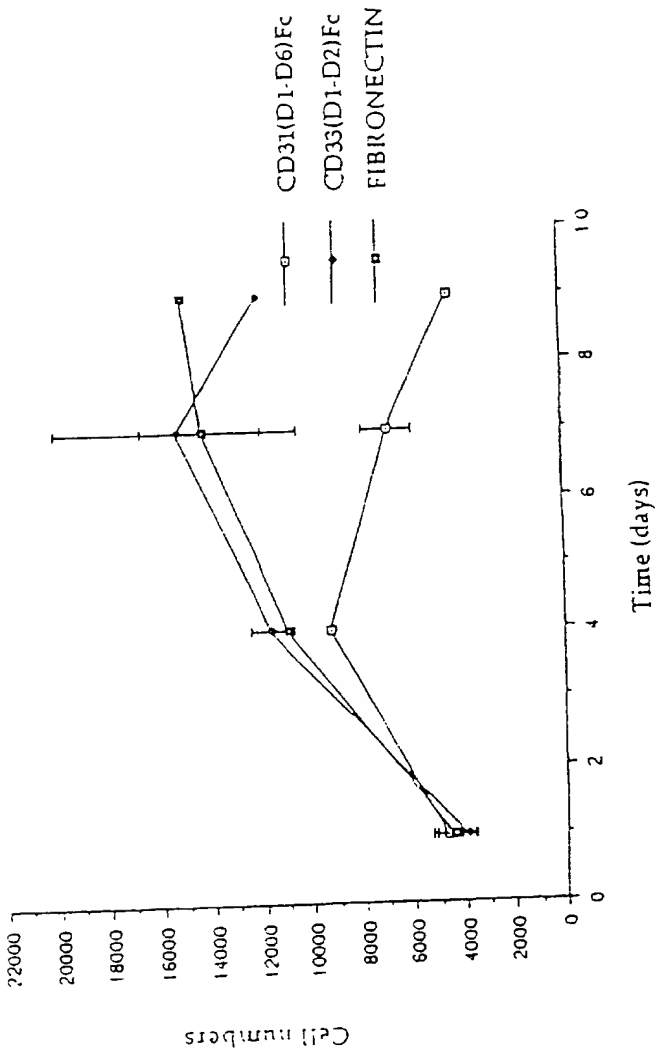

FIGURE 8

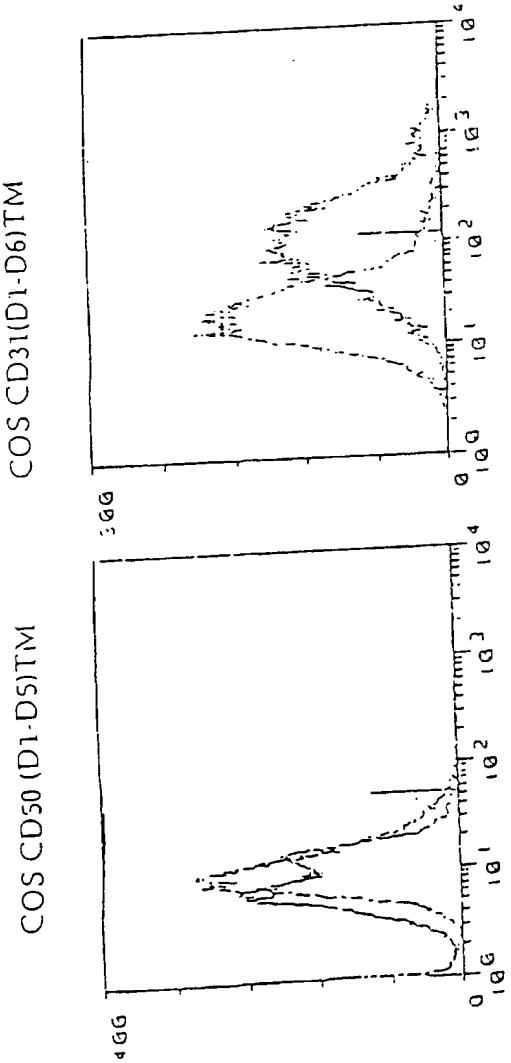

FIGURE 9

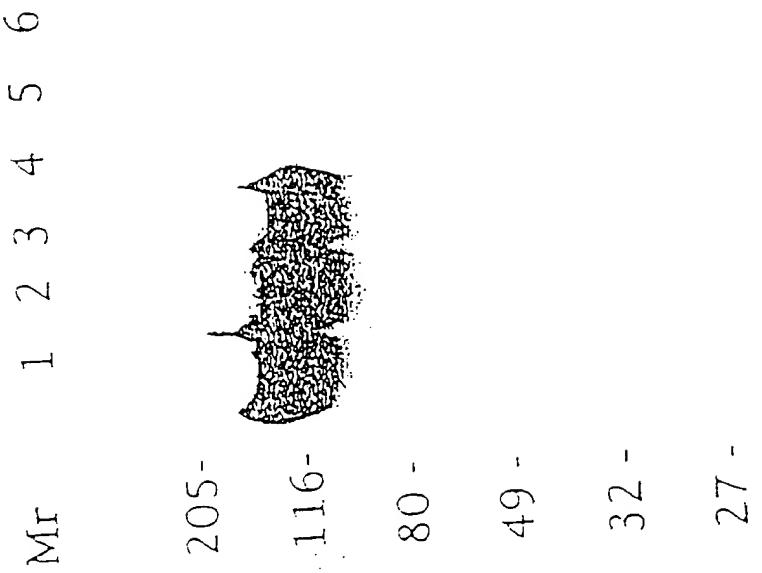

FIGURE 10

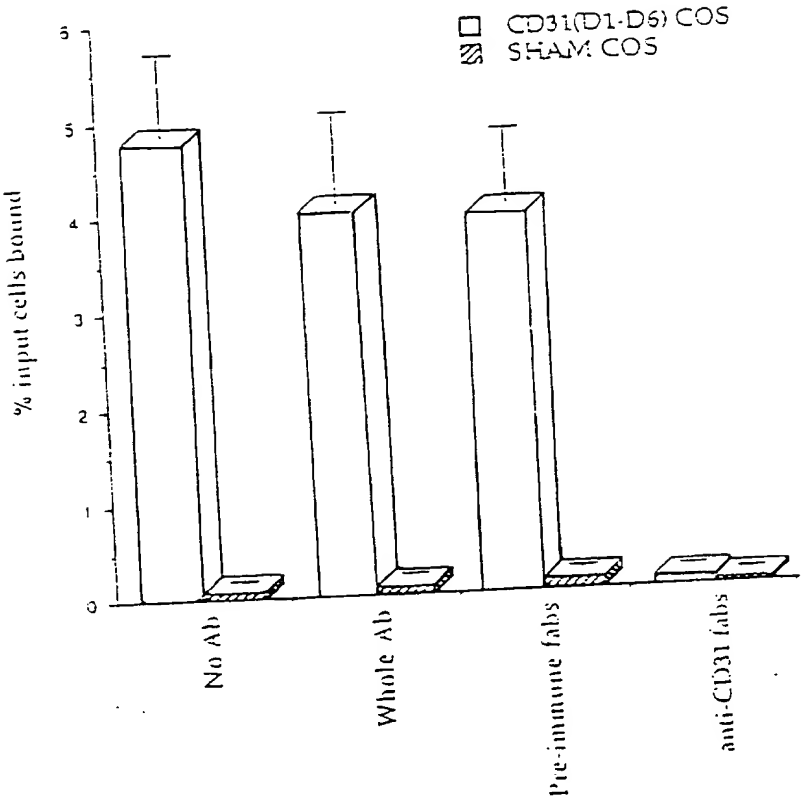

FIGURE 11

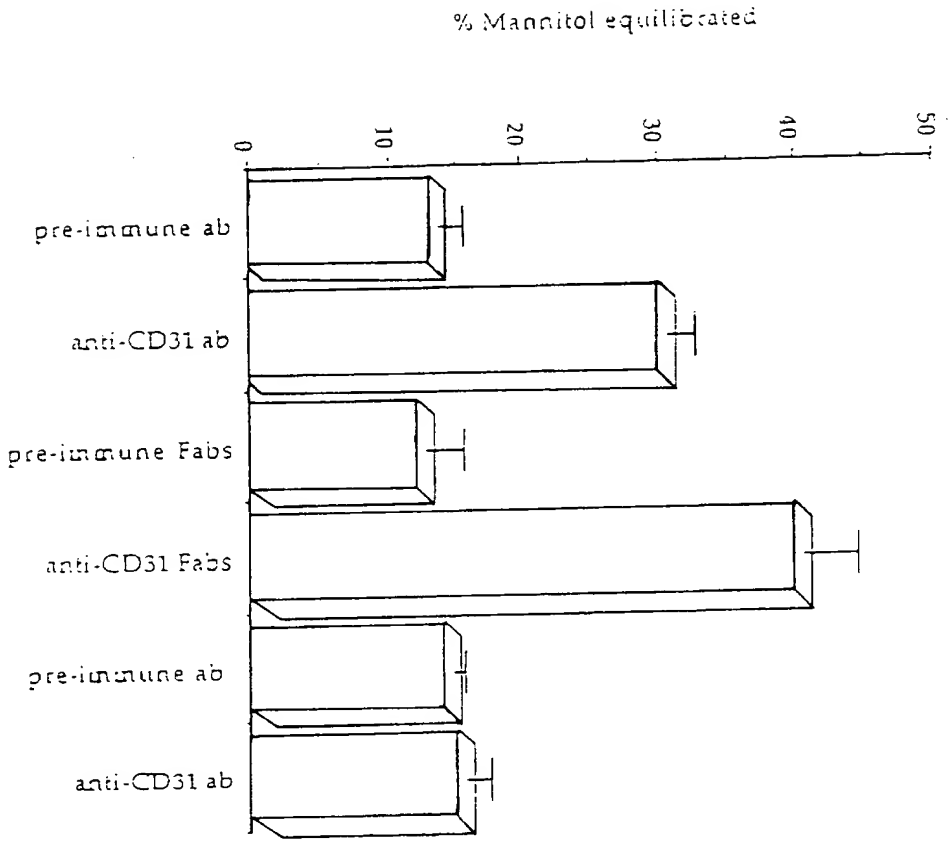

FIGURE 12

Antibody	Fc protein	CD31					MUC18 control
	D1	D1-D2	D1-D3	D1-D4	D1-D5	D1-D6	
9G11	0.235	0.276	0.292	0.291	0.292	0.292	0 050
JC70A	0.29	0.317	0.311	0.317	0.283	0.297	0 052
1.133.1	0.054	0.187	0.208	0.207	0.246	0.224	0 049
CLB/CD31	0.059	0.230	0 242	0.237	0.293	0.249	0 051
5.6E	0.063	0.204	0 224	0.184	0.229	0 207	0 050
HC1/6	0.066	0.069	0 071	0.140	0.243	0.254	0.060
1088	0.071	0.056	0 074	0.088	0 260	0 272	0.060

FIGURE 13

PROTEIN-PROTEIN BINDING ASSAY

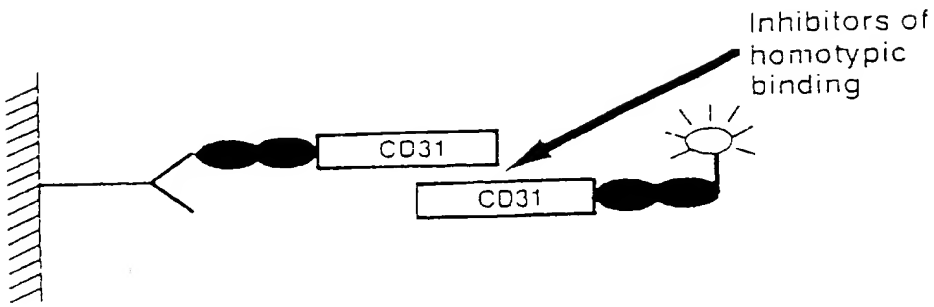

FIGURE 14

PROTEIN-CELL BINDING ASSAY

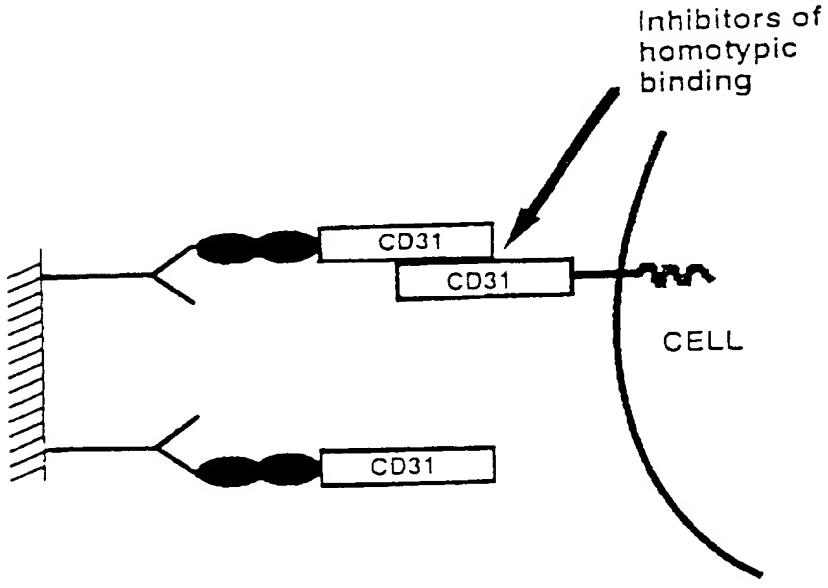

FIGURE 15

FIGURE 16

CD31 DOMAIN SEQUENCES.

All constructs have the signal peptide sequence which is cleaved between G27 and Q 28;

1 MQPRWAQGAT MWLGVLLTLL LCSSLEG 27

Therefore, all mature proteins begin with Q28:

CD31(D1)

+28 QEN
SFTINSVDMK SLPDWTVQNG KNLTLQCFAD VSTTSHVKPQ HQMLFYKDDV
LFYNISSMKS TESYFIPEVR IYDSGTYKCT VIVNNKEKTT AEYQLLVEGV
PSPRV 125

CD31(D1-D2)

+28 QEN
SFTINSVDMK SLPDWTVQNG KNLTLQCFAD VSTTSHVKPQ HQMLFYKDDV
LFYNISSMKS TESYFIPEVR IYDSGTYKCT VIVNNKEKTT AEYQLLVEGV
PSPRVTLDKK EAIQGGIVRV NCSVPEEKAP IHFTIEKLEL NEKMVKLKRE
KNSRDQNFVI LEFPVEEQDR VLSFRCQARI ISGIHMQTSE STKSELVTVT
ESFS 234

CD31(D1-D3)

+28 QEN
SFTINSVDMK SLPDWTVQNG KNLTLQCFAD VSTTSHVKPQ HQMLFYKDDV
LFYNISSMKS TESYFIPEVR IYDSGTYKCT VIVNNKEKTT AEYQLLVEGV
PSPRVTLDKK EAIQGGIVRV NCSVPEEKAP IHFTIEKLEL NEKMVKLKRE
KNSRDQNFVI LEFPVEEQDR VLSFRCQARI ISGIHMQTSE STKSELVTVT
ESFSTPKFHI SPTGMIMEGA QLHIKCTIQV THLAQEFPEI IIQKDKAIVA
HNRHGNKAVY SVMAMVEHSG NYTCKVESSR ISKVSSIVVN ITELFSKPEL
ESSF 334

CD31(D1-D4)

+28 QEN
SFTINSVDMK SLPDWTVQNG KNLTLQCFAD VSTTSHVKPQ HQMLFYKDDV
LFYNISSMKS TESYFIPEVR IYDSGTYKCT VIVNNKEKTT AEYQLLVEGV
PSPRVTLDKK EAIQGGIVRV NCSVPEEKAP IHFTIEKLEL NEKMVKLKRE
KNSRDQNFVI LEFPVEEQDR VLSFRCQARI ISGIHMQTSE STKSELVTVT
ESFSTPKFHI SPTGMIMEGA QLHIKCTIQV THLAQEFPEI IIQKDKAIVA
HNRHGNKAVY SVMAMVEHSG NYTCKVESSR ISKVSSIVVN ITELFSKPEL
ESSFTHLDQG ERLNLSCSIP GAPPANFTIQ KEDTIVSQTQ DFTKIASKSD
SGTYICTAGI DKVVKKSNTV QIVVCEMLSQ PRI 413

FIGURE 16 (Cont'd)

CD31(D1-D5)

```
+28 QEN
SFTINSVDMK SLPDWTVQNG KNLTLQCFAD VSTTSHVKPQ HQMLFYKDDV
LFYNISSMKS TESYFIPEVR IYDSGTYKCT VIVNNKEKTT AEYQLLVEGV
PSPRVTLDKK EAIQGGIVRV NCSVPEEKAP IHFTIEKLEL NEKMVKLKRE
KNSRDQNFVI LEFPVEEQDR VLSFRCQARI ISGIHMQTSE STKSELVTVT
ESFSTPKFHI SPTGMIMEGA QLHIKCTIQV THLAQEFPEI IIQKDKAIVA
HNRHGNKAVY SVMAMVEHSG NYTCKVESSR ISKVSSIVVN ITELFSKPEL
ESSFTHLDQG ERLNLSCSIP GAPPANFTIQ KEDTIVSQTQ DFTKIASKSD
SGTYICTAGI DKVVKKSNTV QIVVCEMLSQ PRISYDAQFE VIKGQTIEVR
CESISGTLPI SYQLLKTSKV LENSTKNSND PAVFKDNPTE DVEYQCVADN
CHSHAKMLSE VLRVKVIAPV DEVQ 504
```

CD31(D1-D6)

```
+28 QEN
SFTINSVDMK SLPDWTVQNG KNLTLQCFAD VSTTSHVKPQ HQMLFYKDDV
LFYNISSMKS TESYFIPEVR IYDSGTYKCT VIVNNKEKTT AEYQLLVEGV
PSPRVTLDKK EAIQGGIVRV NCSVPEEKAP IHFTIEKLEL NEKMVKLKRE
KNSRDQNFVI LEFPVEEQDR VLSFRCQARI ISGIHMQTSE STKSELVTVT
ESFSTPKFHI SPTGMIMEGA QLHIKCTIQV THLAQEFPEI IIQKDKAIVA
HNRHGNKAVY SVMAMVEHSG NYTCKVESSR ISKVSSIVVN ITELFSKPEL
ESSFTHLDQG ERLNLSCSIP GAPPANFTIQ KEDTIVSQTQ DFTKIASKSD
SGTYICTAGI DKVVKKSNTV QIVVCEMLSQ PRISYDAQFE VIKGQTIEVR
CESISGTLPI SYQLLKTSKV LENSTKNSND PAVFKDNPTE DVEYQCVADN
CHSHAKMLSE VLRVKVIAPV DEVQISILSS KVVESGEDIV LQCAVNEGSG
PITYKFYREK EGKPFYQMTS NATQAFWTKQ KASKEQEGEY YCTAFNRANH
ASSVPRSKIL TVRVILAPWK K 601
```

CD31 DOMAIN BOUNDARIES

CD31(D1)	GVPSPRV 125/
CD31(D1-D2)	TVTESFS.234/
CD31(D1-D3)	PELESSF 334/
CD31(D1-D4)	MLSQPRI 413/
CD31(D1-D5)	APVDEVQ 504/
CD31(D1-D6)	ILAPWKK 601/

METHOD OF SCREENING FOR CD31 INTERACTION INHIBITORS

The present invention relates to a method of screening for compounds which inhibit CD31 interactions.

Amino acids and amino acid residues are represented herein by their standard codes as identified by the IUPAC-IUB Biochemical Nomenclature Commission.

CD31, also known as platelet cell adhesion molecule 1 (PECAM 1), is a member of the immunoglobulin superfamily (IgSF). It is a type I integral membrane protein. Its extra cellular domain comprises six Ig C2 related domains (Newman et al., Science. (Wash.DC) 247:1219-1221 (1990); Simmons et al., J.Exp.Med. 171:2147-2151 (1990); Stockinger et al., J.Immunol. 145:3889-3897 (1990)).

CD31 is a surface glycoprotein constitutively and abundantly expressed on the vascular endothelium, with up to one million molecules per cell (Newman and Albelda Nouv.Rev.Fr.Hematol 34:9-13 (1992); Muller et al., J.Exp.Med. 170:399-414 (1989). It is also expressed on platelets (5-8000 per cell), monocytes and neutrophils (100,000 per cell). Approximately 50% of T-cells also express CD31, but at much lower levels than myeloid cells (Stockinger et al., Immunology. 75:53-58 (1992); Bird et al., Immunology 80:553-560 (1993)).

Albelda et al J.Cell Biol. 114:1059-1068 (1991); Muller et al., J.Exp.Med. 175:1401-1404 (1992); deLisser

et al., J.Biol.Chem. 268:16037-16046 (1993); Watt et al., Blood. 82:2649-2663 (1994) have shown that CD31 has both homo- and heterotypic adhesive properties. It has been suggested that the heterotypic binding site is located in CD31 domain 2 which contains a consensus motif for heparan sulphate recognition. CD31 has been recently shown to also bind glycosaminoglycans via this domain. The domain or domains mediating homotypic adhesion have not been identified.

Bevilacqua et al., Annu.Rev.Immunol 11:767-804 (1993) have reviewed research papers relating to members of the IgSF such as intercellular adhesion molecule 1 (ICAM1) and vascular cell adhesion molecule 1 (VCAM1) play key roles in mediating the adhesion of leukocytes to endothelial cells. Albelda et al., J.Cell Biol. 110:227-1237 (1990) have suggested that IgSF members play a role in maintaining inter-endothelial cell contacts. The suggestion is based on work on endoCAM, the bovine homologue of CD31. Polyclonal anti-endoCAM antibodies disrupt bovine adrenal capilliary endothelial contacts.

Leach et al., J.Cell Sci. 104:1073-1081 (1993) and Ayalon et al., J.Cell Biol 126:247-258 (1994) have carried out electron microscopy studies and shown that CD31 is present in inter-endothelial contact areas, but absent from the very tight junction zones.

CD31 has been shown to play a role in the transendothelial migration of monocytes and neutrophils (Muller et al., J.Exp.Med.178, 449-460 (1993)). A monoclonal antibody to CD31 inhibited the random migration of monocytes and neutrophils across an endothelial monolayer, as did a recombinant form of CD31 consisting of domains 1-5 and a half of domain 6. However, the expression of CD31 on lymphocytes is not a pre-requisite for trans-endothelial migration (Bird et

al., Immunogy 80, 553-560 (1993)). Two animal model studies have shown that CD31 is important for neutrophil extravasation in vivo. Firstly, a rabbit antibody raised against human CD31, that cross-reacts with rat CD31, blocks accumulation of rat neutrophils into the peritoneal cavity and the alveolar compartment of the lung (Vaporciyan et al., Science 262, 1580-1582 (1993)). In addition, this reagent inhibited neutrophil accumulation in human skin grafts in immunodeficient mice. Secondly, in a murine model of acute peritonitis, an anti-murine CD31 mab inhibited leukocyte and particularly neutrophil emigration (Bogen et al., J.Exp.Med.179, 1059-1064 (1994)).

The present inventors have shown that homotypic binding of CD31 to CD31 involves inter-digitation of apposed CD31 molecules and specific engagement of binding sites located in domains 2 and 6. Inhibition of CD31 - CD31 interactions can inhibit cell proliferation of endothelial cells. In addition, the permeability of endothelial monolayers is reduced if inhibitors of CD31 - CD31 interactions are present while monolayers are forming; there is no effect once monolayers have formed.

Inhibition of cell endothelial proliferation can be used in the treatment of carcinomas and inflammation, and inhibition of leucocyte recruitment to sites of inflammation appears to involve CD31. It is therefore desirable to be able to efficiently identify inhibitors of CD31 interactions.

Accordingly, the present invention can provide a method of screening for compounds which inhibit CD31 interactions comprising incubating labelled CD31 component with potential inhibitor, adding this mixture to CD31 component immobilised on a support, washing and detecting label.

Alternatively, potential inhibitor can be incubated with CD31 component immobilised on a support and labelled CD31 component can be added followed by washing and detecting label.

CD31 component, hereinafter referred to as CD31C, includes the essential extracellular domains of CD31. Examples of CD31C are extracellular domains 1 to 6 of CD31 and natural CD31, e.g. naturally expressed by myeloid cells.

Preferably, labelled CD31C is incubated separately with potential inhibitor before it is added to CD31C immobilised on a support.

The support can be of any known type for example an ELISA plate, beads, test-tube or membrane such as a nitrocellulose membrane. The support may be manufactured from e.g. glass, polystyrene, PTFE or nylon. Preferably the support is a 96 well plate (Immulon 3, Dynatech, Chantilly, VA).

Preferably the support is coated with antibody A, preferably a goat anti-human Fc antibody. Preferably 1 μg of antibody A is bound to each well. It will be apparent to the man skilled in the art that any anti-human Fc antibody may be used. Coating can be carried out by incubating the support with antibody A at 4°C overnight. The support is preferably washed in phosphate buffered saline (PBS) three times and remaining unbound sites are preferably blocked with PBS/0.4% bovine serum albumin (BSA) (Fraction V, Sigma) by incubating for 2 hours at room temperature.

Preferably domains 1 to 6 of CD31 (CD31 (D1 - D6)) are immobilised on the solid support. Preferably this is carried out by binding a fusion protein comprising

domains 1 to 6 of CD31 and the Fc region of an antibody B, preferably human IgG 1, to antibody A.

Preferably, after PBS/0.4% BSA is added and incubated for 2 hours at room temperature, the chimeric protein (about 5 μg/ml) in PBS is added and incubated for at least about 2 hours at room temperature.

Preferably the fusion protein is such that CD31 domains 1 to 6 are arranged in sequence as in naturally occuring CD31. The Fc region of antibody B is bound directly or via a link to a CD31 domain. This arrangement can ensure the preferred orientation of the CD31 domains; antibody B Fc binds to antibody A and CD31 domains 1 to 6 extend from antibody B Fc away from the support.

The fusion protein comprising antibody B Fc and CD31C can be produced using the following technique:

A full length CD31 (including the transmembrane region) cDNA clone (CD31 (D1-D6) TM) is isolated by transient expression screens of HUVEC cDNA libraries and used as a template for PCR constructs (Simmons et al., J.Exp.Med.171, 2147-2151 (1990)). The PCR strategy for construction of soluble forms can be as follows.

PCR conditions: 4 cycles of : 94°C, 1 minute; 45°C, 2 minutes; 72°C, 2 minutes; followed by 30 cycles of 94°C, 30 seconds; 45°C, 30 seconds; 72°C, 1.5 minutes. Primers used can be : pCDM8 forward amplification primer plus reverse amplification primers containing the common adaptor sequence 5'GATCAGATCTACTTACCTGT plus domain specific sequences as follows :

domain 1, CACCCTGGGACTGGGCACTCC
domain 2, AGAGAAGGATTCCGTCACGGT
domain 3, GAAGGAAGATTCCAGTTCGGG

domain 4, AATCCTGGGCTGGGAGAGCAT
domain 5, CTGGACCTCATCCACCGGGGC
domain 6, TTTCTTCCATGGGGCAAGAAT.

PCR products were cut with HindIII and BglII and cloned into pIg vector (Fawcett et al., Nature 360, 481-484 (1992)), Simmons, Cellular Interactions In Development, 93-128 (1993)) cut with HindIII and BamHI.

The resulting plasmid (fusion plasmid) can express a fusion protein comprising CD31C and the Fc region of Ig.

The fusion plasmid is transfected into COS cells (10μg/10^7 COS cells) using diethyl aminoethyl-dextran (DEAE-dextran) as a facilitator (Simmons, Cellular Interactions In Development, 93-128 (1993)). The medium is changed at 24 hours to DMEM/0.5% FCS and supernatant harvested at 7 days. Fusion proteins are affinity isolated on Protein A Sepharose (TM); columns are washed with 0.1 M glycine pH 5.0 (to remove bovine IgG) and Fc chimaeras eluted with 0.1 M glycine pH 3.0, neutralised immediately in 10% v/v 1 M Tris base, buffer exchanged and concentrated by centrifugal dialysis (Centricon 10 units, Amicon).

Labelled CD31C preferably comprises at least domains 1 to 6 of CD31 and a label. The label can be any known label; it is preferably a radio-active or fluorescent cell. The cells can be produced by, for example, the following procedure:

COS cells are transfected with the fusion plasmid described above and labelled by incubating for 24/48 hours with [^{3}H]-thymidine(10μCi/2x10^7 cells). Transfection efficiency is checked by cytofluorography and is usually 15-30%. Cells are lifted with PBS/2mM

EDTA, washed three times in RPMI-1640 and resuspended in assay buffer (RPMI-1640, 0.2% BSA) at 3-4x10^4 cells/well.

The cells are preferably CD31 transfected COS cells, although it will be apparent to those skilled in the art that other transfected cells for example L and CHO cells, and cells which naturally express CD31 such as MIKALL, U937 and H82 cells can be used. Preferably the cells are COS-1 or COS-7 cells.

Preferably potential CD31 interaction inhibitors are incubated with the cells at room temperature for about 10 minutes before incubating this mixture with CD31 bound to support.

To ensure that CD31 domain 2 heterotypic interactions are blocked, assays can be repeated in the presence of 100 μg/ml heparin. This concentration of heparin has been shown to block the heterotypic binding of domain 2 to heparan sulphate decorated proteoglycans.

Washing can be carried out using the assay buffer (described above) to remove unbound cells.

Detection of label can be carried out using known techniques. For example, if the label is a radioactive cell, detection can be carried out by scintillation counting.

The more label bound to the support detected, the less effective the binding inhibitor.

Using a deletion series as shown in Figure 16 it is possible to determine a CD31 domain with which a CD31 interaction inhibitor reacts. This can be carried out by preparing a series of CD31 deletion variants having e.g. domain 1, domains 1 and 2, domains 1 to 3, 1 to 4, 1 to

5, and 1 to 6, and carrying out the method of the invention wherein the deletion variants are CD31C immobilised on a support. The minimum number of domains required to react with a CD31 interaction inhibitor can indicate a domain with which the inhibitor reacts. For example, if an inhibitor reacts with deletion variants having domains 1 to 4, 1 to 5 and 1 to 6, but not with those having domain 1, domain 1 and 2, and domains 1 to 3, it can be concluded that at least part of domain 4 reacts with the inhibitor. Accordingly, the method can be used to map regions of CD31 in which interactions take place. This can be carried out as in Example 2.

The invention is now described by reference to the accompanying figures of which :

FIGURE 1 is a diagrammatic representation of full length membrane expressed CD31(CD31(D1-D6)TM) and CD31(D1-D6)-Fc.

FIGURE 2 represents the results of adhesion of COS cells transfected with CD31(D1-D6)TM, sham (pCDM8) transfected COS cells, U937 (promonocytic, $CD31^+$), and H82 (small lung cell carcinoma, $CD31^-$) to CD31(D1-D6)-Fc immobilised on plastics material. Assays are expressed as mean % of total input cells binding +/- 1 SD (n=6).

FIGURE 3 represents the results of adhesion of CD31(D1-D6)TM^+COS transfectants to plastic coated with a C-terminal domain deletion series of CD31-Fc proteins. The series used was; CD31(D1)Fc; CD31(D1-D2)Fc; CD31(D1-D3)Fc; CD31(D1-D4)Fc; CD31(D1-D5)Fc; CD31(D1-D6)Fc. Adhesion to control protein, MUC18-Fc, is included as a negative IgSF-Fc control. Results are expressed as the means of the % CD31(D1-D6)TM^+COS cells binding +/- 1 SD (n=6) i.e. corrected for transfection efficiency.

FIGURE 4 represents results of adhesion assays of CD31(D1-D2)TM and CD31(D3-D6)TM transfectants to CD31(D1-D6)-Fc. Background adhesion of all COS transfectants to CD14-Fc are also shown. Results are expressed as the means of the % CD31⁺COS cells binding +/- SD (n=6). All assays are normalised with respect to % transfection efficiency.

FIGURE 5 represents the results of a screen of 7 anti-CD31 mabs for blockade of adhesion of CD31(D1-D6)TM⁺COS to CD31(D1-D6)Fc. Mabs were present during the assay. None denotes results from wells where no antibody was incubated with the cells. MUC18Fc was used as a negative control.

JC70A was produced according to the procedure of Parums et al., J.Clin.Path.43, 752-757 (1990); MUC-18 was produced according to the procedure of Fawcett et al., Nature 360, 481-484, (1992); 9G11 and 10B8,, L133.1, 5.6E, CLB/CD31 and CH1/6 are commercially available from British Biotechnology, Becton Dickinson, Immunotech, Monosan and Serotec respectively.

Cells were incubated with mabs at 10 μg/ml for 10 minutes at room temperature before the assay. Mabs were present during the asay. MUC18-Fc is a negative control.

FIGURE 6 represents results of competitive blockade of adhesion of CD31(D1-D6)TM⁺COS to CD31(D1-D6)Fc by CD2(D1-D2)Fc (negative control), CD31(D1)Fc, and CD31(D1-D2)Fc. Chimaeric Fc competitor proteins were included at 50 μg/ml 10 minutes before, and during the assay. The background level of binding in this assay is indicated by adhesion of CD31(D1-D6)TM⁺COS to MUC18-Fc.

FIGURE 7 represents a dose response curve of CD31(D1-D6)Fc. HUVEC were seeded at a subconfluent

density on surfaces precoated with CD31(D1-D6)Fc or CD33(D1-D2)Fc over a range of concentrations. Cells were seeded on day 0 at 2 x 10^4 cells/well. Total cell numbers were counted at day 5. Results are presented as means of duplicate wells.

FIGURE 8 represents growth rate curves of HUVEC on surfaces coated with fibronectin alone, or fibronectin plus 100 μg/ml of CD33(D1-D2)Fc of CD31(D1-D6)Fc. Cells were seeded on day 0 at 10^3 cells/well and cell numbers measured by counting. Results are expressed as mean +/- 1 SD (n=4).

FIGURE 9 represents characterisation of polyclonal anti-CD31 antibodies by FACScan analysis of CD31(D1-D6)TM⁺COS and CD50(ICAM-3) transfected COS using IgG fraction from pooled serum at 1:1000 dilution. Control background staining of pre-immune rabbit IgG is shown in each panel.

FIGURE 10 represents characterisation of polyclonal anti-CD31 antibodies by immunoprecipitation from surface [^{125}I]-labelled HUVEC. Lane 1, polyclonal anti-CD31 whole serum; lane 2, polyclonal anti-CD31 IgG fraction; lane 3, anti-CD31 mab 9G11; lane 4, pre-bleed serum; lane 5, pre-bleed IgG1; lane 6, anti-ICAM-3 (CD50) mab CH3.3. Molecular masses are indicated at left in kDa.

FIGURE 11 represents adhesion of CD31(D1-D6)TM⁺COS or mock transfected COS to CD31(D1-D6)Fc immobilised on plastic in the absence or presence of anti-CD31 polyclonal antibodies (whole antibody, or Fabs). All antibodies were present throughout the adhesion assay and used at 50μg/ml. Results are expressed as means +/- 1 SD (n=8).

FIGURE 12 represents typical [^{14}C]-mannitol

equilibration results 1-4 represents typical [^{14}C]-mannitol equilibration results from HUVEC monolayers formed on Transwells in the presence of CD31 and pre-immune (PI) antisera or Fab fragments (50 μg/ml). Significant increases in monolayer permeability were observed when HUVEC were incubated with either CD31 antisera or Fab fragments during monolayer formation ($p<0.02$).

The 5th and 6th columns show typical [^{14}C]-mannitol equilibration results from pre-formed HUVEC monolayers incubated overnight with pre-immune or CD31 antisera. No significant difference in equilibration values could be detected ($p<0.24$). All monolayers were tested prior to the incubation and gave percentage equilibration values within the normal range.

FIGURE 13 represents results of ELISA assays of the 7 anti-CD31 mabs referred to in Figure 5. Chimeric CD31-Fc proteins were immobilised via goat anti-human IgG1Fc and screened with anti-CD31 mabs. MUC18-Fc was included as a negative control IgSF-Fc chimaera. Results are means of duplicates and representative of two separate experiments.

FIGURE 14 is a schematic of the screen used in the method of the present invention.

FIGURE 15 is a schematic of the screen used in the method of the present invention wherein the label comprises a labelled cell which expresses CD31.

FIGURE 16 shows the amino acid sequence of CD31(D1), CD31(D1-D2), CD31(D1-D3), CD31(D1-D4), CD31(D1-D5) and CD31(D1-D6).

The invention is now described with reference to the

following illustrative Examples.

EXAMPLE 1

Polyclonal anti-CD31 antisera were raised in rabbits using CD31(D1-D6)Fc as an immunogen. The specificity of these reagents were established in two ways. Firstly, the affinity purified IgG fraction from these sera specifically recognised CD31(D1-D6)TM$^+$COS transfectants (Figure 9) and secondly they precipitated a single 130 kD protein from [^{125}I] surface labelled endothelial cells (Figure 10).

The effect of the polyclonal anti CD31 antibody was tested to determine whether it blocked homotypic binding of CD31. In order to avoid the effects of cross-bridging of CD31(D1-D6)TM molecules expressed on the COS cells to CD31(D1-D6)Fc bound to the plate, Fab fragments were prepared. Pre-incubation of CD31$^+$COS transfectants with 50 μg/ml Fab fragments of the anti-CD31 antibodies resulted in reduction of CD31-CD31 adhesion to background levels (see Figure 11). Thus anti-CD31 Fab fragments can block homotypic CD31-CD31 adhesion.

EXAMPLE 2

A panel of anti-CD31 monoclonal antibodies (mabs) is screened for their ability to block adhesion. COS 7 cells were transfected with pCDM8 plasmid containing full length transmembrane CD31. Cells were labelled for 24 hours with [^{3}H]-thymidine (10μCi/2 x 10^7 cells). Transfection efficiency was checked by cytofluorography using 2 anti-CD31 antibodies (clone numbers 9G11 and 10B8) and in this experiment was found to be 20%.

Transfected cells were lifted from tissue culture plastic

with PBS/2mM EDTA and washed three times in RPMI 1640. After resuspension in assay buffer (RPMI 1640, 20 mM HEPES, 0.2% BSA) at 6 x 10^5/ml, cells were incubated for 10 minutes at 37°C with anti-CD31 antibodies (final concentration 10μg/ml). 50 μl of cells were then added to wells in the adhesion assay plate and incubated for 2h at 37°C. Six replicates were set up for each antibody or control condition. Antibodies were preent during the assay.

The adhesion assay plates (96 well, Immunion 3, Dynatech, Chantilly, VA) were prepared by pre-coating wells with 1μl/well of affinity purified goat anti-human Fc antibody (Sigma, Poole, Dorset, UK) overnight at 4°C. After washing plates 3 times in PBS and blocking unbound sites with PBS/0.4% BSA for 2 hours at room temperature, 50μl of recombinant CD31(D1-D6)Fe protein at 10μg/ml was added to each well and incubated overnight at 4°C. MUC18Fc was used as a negative control at the same concentration. Plates were then washed twice in PBS and once in assay buffer.

At the conclusion of the incubation, the wells were washed twice with prewarmed assay buffer, using a continuous flow of assay buffer at a head of pressure of 30 cm of water using a siphon arrangement. Between washes the wells were emptied by inversion. Cells that remained bound after two washes were lysed in 1% SDS, scintillant added (Ready Safe, Beckman) and incorporated radioactivity counted using a Beckman LS5000 CE counter.

Results (corrected for transfection efficiency) are expressed in Figure 5. Two the the three mabs (clones 1.133.1 and 5.5E) that map to domain 2 block the binding of COS cells expressing CD31 (CD31·COS) to a fusion protein comprising domains 1 to 6 of CD31 and the Fc region of human IgG1 (CD31(D1-D6)Fc). None of the other

mabs inhibit CD31-CD31 binding in this assay. In fact, as all the mabs used are whole antibodies, there is an actual increase in adhesion probably due to cross bridging of CD31 on the COS cells to CD31 on the assay surface.

The blocking assay can be repeated on a CD31$^+$ lymphoid cell line (MIKALL), with similar results; again the only two blocking mabs are 5.6E and L133.1. The importance of domain 2 in CD31-CD31 interactions can be confirmed using CD31-Fc chimaeric fusion proteins themselves as competitive blocking reagents. CD31(D1-D2)Fc protein fully competes with CD31$^+$COS for binding to CD31(D1-D6)Fc. In addition, CD31(D1)Fc partially competes in this assay (suggesting that domain 1 may play a role in homotypic adhesion).

EXAMPLE 3

A membrane expressed form of domains 3-6 (CD31(D3-D6)TM) was made as follows.

The signal peptide of CD33 (Simmons and Seed J. Immunol 14, 2997-2800 (1988)) was isolated as a 72 bp HindIII/BamHI fragment and cloned into a pcDNAIneo (invitrogen, San Diego, US), and designated pSig. The PCR product encoding domains 3-6 plus transmembrane domain and cytoplasmic tail was cut with Bgl II and ligated into BamHI cut pSig. Inserts were checked for correct orientation. CD31(D3-D6)TM is missing the C-terminal 36 residues of the cytoplasmic tail. PCR conditions were the same as for generation of soluble deletion series. The primer sequences for generation of CD31(D3-D6)TM were as follows; forward amplification primer, GATC AGATCTG AAG TTC CAC ATC AGC CCC ACC; reverse amplification primer.

C L A I M S :

1. A method of screening for inhibitors of CD31 interactions which comprises incubating labelled CD31 component with potential inhibitor, adding this mixture to CD31 component immobilised on a support, washing and detecting label.

2. A method of screening for inhibitors of CD31 interactions which comprises incubating potential inhibitor with CD31 component immobilised on a support, adding labelled CD31 component, washing and detecting label.

3. A method according to claim 1 or 2 wherein labelled CD31 component comprises domains 1 to 6 of CD31.

4. A method according to claim 3 wherein labelled CD31 component further comprises the Fc region of an immunoglobulin.

5. A method according to claim 4 wherein the immunoglobulin is human IgG.

6. A method according to any preceding claim wherein labelled CD31 component comprises a fusion protein of domains 1 to 6 and the Fc region of human IgG, and a cell which expresses the fusion protein.

7. The method of claim 6 wherein the cell is a COS cell.

8. The method of claim 6 or 7 wherein the cell is radioactively or fluorescently labelled.

9. A method according to any preceding claim wherein the support is solid.

10. A method according to any preceding claim wherein CD31 is immobilised on a support by coating the support with antihuman antibody and then incubating it with a fusion protein of domains 1 to 6 of CD31 and the Fc region of a human immunoglobulin.

11. A method according to any preceding claim wherein washing is carried out with assay buffer comprising RPMI1640 and 0.2% BSA.

12. A method of screening for inhibitors of CD31 interactions and mapping their site of reaction which comprises the method of claim 1 or 2, followed by preparing a series of CD31 extracellular domain deletion variants and repeating the method of claim 1 or 2 wherein the variants are immobilised separately on supports as CD31 components.

13. A method of mapping the site of reaction on CD31 of an inhibitor of CD31 interactions which comprises preparing a series of CD31 extracellular domain deletion variants, immobilising the variants separately on supports, adding labelled CD31 component incubated with inhibitor, washing and detecting label.

14. A method of mapping the site of reaction on CD31 of an inhibitor of CD31 interactions which comprises preparing a series of CD31 extracellular domain deletion variants, immobilising the variants separately on supports, incubating them with inhibitor, adding labelled CD31 component, washing and detecting label.

17

Patents Act 1977
Examiner's report to the Comptroller under Section 17
(The Search report)

Application number
GB 9421118.2

Relevant Technical Fields

(i) UK Cl (Ed.N) G1B (BAD,BAE,BAG,BAX)

(ii) Int Cl (Ed.6) G01N 33/577,33/68

Search Examiner
DR N CURTIS

Date of completion of Search
10 JANUARY 1995

Databases (see below)
(i) UK Patent Office collections of GB, EP, WO and US patent specifications.

(ii) ONLINE: WPI, BIOTECH/DIALOG

Documents considered relevant following a search in respect of Claims :-
1-14

Categories of documents

X: Document indicating lack of novelty or of inventive step.

Y: Document indicating lack of inventive step if combined with one or more other documents of the same category.

A: Document indicating technological background and/or state of the art.

P: Document published on or after the declared priority date but before the filing date of the present application.

E: Patent document published on or after, but with priority date earlier than, the filing date of the present application.

&: Member of the same patent family; corresponding document.

Category	Identity of document and relevant passages		Relevant to claim(s)
A	WO 94/15641 A1	(ROCKEFELLER UNIVERSITY) see page 10, line 29 to page 30, line 16	1
A	WO 91/10683 A1	(BLOOD CENTER OF SOUTHEAST WISCONSIN) see page 20, lines 3-6	

Databases: The UK Patent Office database comprises classified collections of GB, EP, WO and US patent specifications as outlined periodically in the Official Journal (Patents). The on-line databases considered for search are also listed periodically in the Official Journal (Patents).